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Original Article

Increased SUMO-2/3-ylation mediated by SENP3 degradation is protective against cadmium-induced caspase 3-dependent cytotoxicity

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ABSTRACT — Increased post-translational modification of proteins by SUMO-2/3 is a cytoprotective response against cell stress induced by ischaemia and reperfusion. However, it is still unclear what other cell stressors trigger protein SUMOylation, what mechanisms enhance and maintain the enhanced SUMOylation, and if it is a general protective mediator against other cytotoxic stresses. Here, we show increased levels of SUMOylation and decreased levels of the SUMO deconjugating enzyme SENP3 in PC12 cells treated with the toxic heavy metal cadmium. In addition, SENP3 knockdown reduced cadmium-induced caspase 3 cleavage and cell death in PC12 cells, while SENP3 overexpression enhanced cell death. These results suggest that SENP3 is an important regulator of the cellular response to cadmium stress in PC12 cells. Our findings are consistent with previous reports of decreased SENP3 and increased SUMOylation in ischaemia, and imply that the regulation of SENP3 levels and subsequent changes in SUMOylation could be a cytoprotective mechanism against caspase 3-mediated cell death.

Key words: Cadmium, Apoptosis, Cell stress, SUMOylation, SENP3

INTRODUCTION

Cadmium is a heavy metal and environmental pollutant arising mainly from the burning of fossil fuels and incineration of municipal waste. Chronic exposure to cadmium leads to its accumulation in various tissues and resultant toxicity. In particular, cadmium promotes mitochondrial dysfunction (Xu *et al.*, 2013) and can elicit mitochondrial-dependent apoptosis (Adiele *et al.*, 2011). More specifically, cadmium inhibits the function of electron transport chain complexes, resulting in mitochondrial uncoupling via mitochondrial permeability transition, increased generation of ROS (Templeton and Liu, 2010) and oxidative stress (Adiele *et al.*, 2011; Onukwufor *et al.*, 2014).

Small ubiquitin-like modifier (SUMO) is a 97-residue protein that is covalently conjugated to specific lysine residues on target proteins to modify their function.

SUMOylation is a critically important control process in all eukaryotic cells because it acts as a biochemical switch to regulate the function of hundreds of proteins in many different pathways. Although the molecular consequences of SUMOylation are varied, the underlying principle is that it alters the inter- and/or intramolecular interactions of substrate proteins to change their localisation, stability, and/or activity (Wilkinson and Henley, 2010).

There are three SUMO paralogues (SUMO-1-3) in mammals. SUMO-2 and SUMO-3 are identical except for three residues but share only ~50% sequence identity with SUMO-1 (Wilkinson and Henley, 2010). SUMO conjugation occurs via a three-step enzymatic pathway analogous to protein ubiquitination. SUMO is first 'activated' for conjugation by an E1 enzyme, a heterodimer of SAE1 and SAE2 in humans, and then passed to the conjugating enzyme Ubc9. Ubc9, often in conjunction with

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an E3 enzyme, covalently attaches SUMO to the substrate protein (Wilkinson and Henley, 2010). SUMO is efficiently removed from substrate proteins by SUMO proteases, making SUMOylation a reversible and highly dynamic modification. Nine mammalian SUMO proteases (SENP1-3, SENP5-7, DESI1, DESI2 and USPL1) have been reported. Of these, DESI1, DESI2 and USPL1 have only recently been discovered and little is known about them (Hickey *et al.*, 2012). Individual SENPs display different subcellular localisations and SUMO paralogue specificity (Guo and Henley, 2014).

We, and others, have shown that global increases in SUMO-2/3 conjugation are a cellular protective response to severe ischaemic stress (Datwyler *et al.*, 2011; Cimarosti *et al.*, 2012; Lee *et al.*, 2016; Lee *et al.*, 2007). The unfolded protein response (UPR) mediates protective responses that allow cell adaptation and survival (Hetz, 2012). We have shown that the SUMO-2/3-specific protease SENP3 is degraded during ischaemia, via a pathway involving the unfolded protein response (UPR) kinase PERK and the lysosomal enzyme cathepsin B. A key target for SENP3-mediated deSUMOylation is the GTPase Drp1, which plays a major role in regulating mitochondrial morphology and integrity (Zungu *et al.*, 2011). Under conditions of extreme cell stress recruitment of Drp1 to the mitochondria causes, among other things, fragmentation and cytochrome *c* (cyt *c*) release, which are potent signals for cell death (Chang and Blackstone, 2010). Depletion of SENP3 prolongs Drp1 SUMOylation, which prevents mitochondrial disruption and protects from cell death. SENP3 levels recover following reoxygenation after ischaemia allowing deSUMOylation of Drp1, which facilitates Drp1 localization to mitochondria and promotes fragmentation, cytochrome *c* release and eventual cell death. RNAi knockdown of SENP3 protects cells from reoxygenation-induced cell death via a mechanism that requires Drp1 SUMOylation (Guo *et al.*, 2013, 2017; Anderson and Blackstone, 2013; Schuldt, 2013). This represents a novel adaptive pathway to extreme cell stress in which dynamic changes in SENP3 stability and regulation of Drp1 by SUMO-2/3 are crucial determinants of cell fate, opening up possibilities of developing new drug targets and therapeutic strategies (Lee *et al.*, 2016; Wei *et al.*, 2012; Yang *et al.*, 2015a, 2015b; Yu *et al.*, 2015).

In this study, we investigated the effect of RNA interference-mediated SENP3 knockdown and SENP3 overexpression on the vulnerability of PC12 cells to cadmium exposure. Our results indicated that SENP3 levels and consequent regulation of protein SUMOylation are critical factors in controlling cell fate in response to cadmium-induced toxicity. These results elucidate a novel adaptive

pathway to extreme cell stress in which dynamic changes in SENP3 levels and regulation of SUMOylation are crucial determinants of cell fate. Additionally, the data suggest possible new drug targets and therapeutic strategies for diseases characterised by extreme oxidative stress.

MATERIALS AND METHODS

Cell culture

Rat pheochromocytoma (PC12) cells and HEK293 cells were grown in RPMI-1640 Medium (Sigma-Aldrich®, St. Louis, MO, USA) or Dulbecco's modified Eagle's medium (DMEM; Lonza, Verviers, Belgium), respectively, both of which were supplemented with 10% foetal bovine serum, 2 mM L-glutamine, 100 units/mL penicillin and 100 µg/mL streptomycin, at 37°C in humidified air supplemented with 5% CO₂.

DNA transfection and RNAi knockdown

PC12 cells were plated onto collagen-coated 12-well plates and when ~50% confluent they were transfected with 0.5 µg DNA to 0.15 mL un-supplemented RPMI-1640 Medium and combined with 0.15 mL un-supplemented medium containing 0.75 µL Lipofectamine® 2000 Transfection Reagent (Invitrogen™, Paisley, UK). This mixture was incubated at room temperature for 20 min, and the cells were washed with un-supplemented medium before the transfection mixture was applied for 2 hr with incubation at 37°C. After this period, the transfection mixture was replaced with complete medium. Cells were then incubated for another 24 hr prior to cadmium exposure.

SENP3 was knocked down in PC12 cells by transient transfection with an shRNA plasmid vector based on HuSH-29 pGFP-V-RS system (Origene Technologies, Inc., Rockville, MD, USA), containing the following sequences:

shRNA1: TGTGGACATCTTCAATAAGGAACTATTGC;
shRNA2: TATGGACA- GAACTGGCTCAATGACCAGGT;
shRNA3: CTTGTCTCAGTTGATGTAAGGCGACGCAC;
shRNA4: ACTGGCTCAATGACCAGGTGATGAACATG.

These sequences targeted rat SENP3 gene transcripts, while an empty pGFP-V-RS vector and a pGFP-V-RS containing a scrambled shRNA sequence were used as negative controls. Successful transfections were determined by GFP reporter gene expression in at least 40% of the total cell population.

HEK293 cells were plated onto 12-well plates and when ~50-70% confluent they were transfected with DNA or siRNA using jetPEI or INTERFERin™ (Polyplus Transfection, Illkirch-Graffenstaden, France),

respectively, as described previously (Guo *et al.*, 2013, 2017). siRNA duplexes used were as follows: human SENP3 siRNA (sc-44451; Santa Cruz Biotechnology, Heidelberg, Germany), and non-specific siRNA (Eurofins MWG Operon, Ebersberg, Germany).

Western blotting and analysis

Cells were harvested by cooling the plates on ice, suspending the adherent cells with a cell scraper, combining the cell suspensions from triplicate wells, centrifuging at 1000 rpm, 4°C for 10 min, and resuspending the cell pellets in 0.4 mL 1 x SDS sample buffer [50 mM Tris-Cl pH 6.8, 2% (w/v) SDS, 10% (v/v) glycerol, 5% (v/v) β -mercaptoethanol, 0.02% (w/v) bromophenol blue]. The cell lysates were briefly sonicated and incubated at 37°C for 10 min before storing at -20°C. After thawing, protein was detected and quantified by western blot. Cell lysates were subjected to SDS-PAGE using 12% polyacrylamide gels and transferred to Immobilon™-FL PVDF Membrane (Millipore, Billerica, MA, USA). After transfer, the membranes were probed with SUMO-2/3 (Rabbit monoclonal; 1:500, Cell Signaling Technology), SENP3 (Rabbit monoclonal 1:1000, Cell Signaling Technology), caspase 3 (Rabbit polyclonal; 1:1000, Cell Signaling Technology, Danvers, MA, USA), GFP (Rabbit polyclonal; 1:2000; Santa Cruz Biotechnology) or β -actin (Mouse monoclonal; 1:10,000; Sigma-Aldrich) primary antibodies and then with anti-rabbit or -mouse HRP conjugated secondary antibodies. Immunoreactive bands were visualised with Luminata Forte Western HRP Substrate (Millipore) and exposure to X-ray film. Developed blots were digitally captured at a resolution of 600 dpi using a Canon PIXMA MG3150 scanner. Specific protein product band signal intensities were determined using ImageJ (NIH). Raw data was processed using Microsoft Excel, with each product signal divided by the corresponding β -actin signal from the same blot, to give relative protein levels which controlled for variations in total protein concentration between samples.

LDH cell death assays

Cell death was measured using the *In vitro* Toxicological Assay Kit, Lactic Dehydrogenase based (Sigma-Aldrich®), according to the recommended procedure, with some modifications. Reactions were performed in duplicate for each sample and carried out in clear 96-well plates. A total of 10 μ L of medium sample was mixed with 50 μ L LDH Assay Mixture per reaction, before sealing from light and incubating at room temperature for 30 min. Following this, 6.7 μ L 1 M HCl was added to each well to terminate the reactions, and the plates were

analysed in a Tecan Sunrise™ microplate reader. This was set to measure the spectrophotometric absorbance at 492 nm after subtracting background absorbance at 620 nm, giving values proportionate to the concentration of LDH in each sample. This data was processed using Microsoft Excel. Duplicate well values were averaged before obtaining average values for each treatment, which was carried out in triplicate. These values were then normalised against the average absorbance in the untreated scrambled wells, which was arbitrarily assigned a relative LDH release of 1.

Statistics

All statistical analyses were conducted by using either Student's t-test with two-tail P-value or one-way analysis of variance followed by Dunn's or Bonferroni post-test as appropriate. All values are presented as mean \pm S.E.M and are normalised to the control value.

RESULTS AND DISCUSSION

To evaluate the dose and time course profile of cadmium, we challenged PC12 cells with 10 μ M or 50 μ M CdCl₂ for up to 8 hr. Surprisingly, these treatments did not induce cell death as substantial cleavage of caspase 3 or release of LDH was not detected (Fig. 1A, B, C). However, a 24-hr exposure to 50 μ M CdCl₂ resulted in significant cell death, which was determined by LDH assays (Fig. 1A).

SUMOylation has been strongly implicated in response to ischaemic stress (Lee *et al.*, 2007, 2016; Yang *et al.*, 2008; Datwyler *et al.*, 2011), so we next investigated if protein SUMOylation levels were altered in PC12 cells in response to cadmium toxicity. As expected, the total SUMO2/3-conjugated protein was increased after exposure to cadmium (Fig. 1D), although the stress was not fatal to the cells. We hypothesized that cadmium stress could enhance SUMOylation by either increasing SUMO conjugation or decreasing SUMO deconjugation. Ischaemic stress has been shown to promote degradation of the deSUMOylation enzyme SENP3 to enhance SUMOylation (Guo *et al.*, 2013). Therefore, we investigated the effect of cadmium on SENP3. As shown in Fig. 1E, SENP3 levels decreased as early as 4-hr after addition of cadmium. These results suggest that degradation of SENP3, most likely resulting in the observed increase in protein SUMOylation, may be protective against cadmium cytotoxicity in the early phase of cell stress.

We subsequently carried out an shRNA knockdown experiment to determine whether the loss of SENP3 and the increased SUMOylation is protective. Four shRNA sequences were screened for their knockdown efficiency,

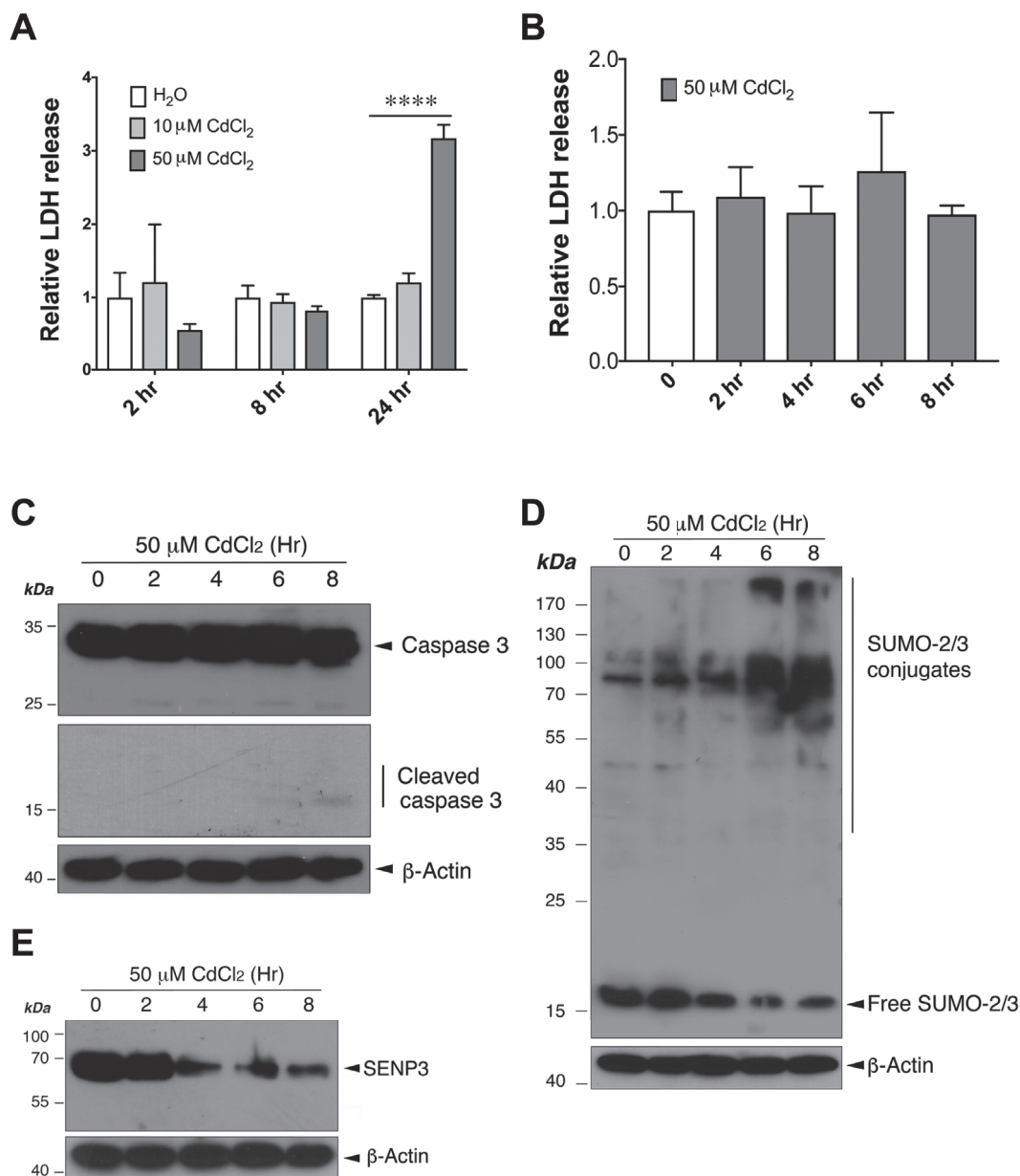


Fig. 1. Short-term cadmium exposure decreases SENP3 and increases global SUMO-2/3 conjugation in PC12 cells. **(A)** Histogram showing changes in LDH levels in conditioned culture media prepared from PC12 cells treated with 10 μM or 50 μM CdCl₂ for the indicated durations. The results are presented as mean ± S.E.M. (n = 3 replicates for each group, ****p < 0.0001; one-way analysis of variance). **(B)** Histogram showing changes in LDH levels in conditioned culture media prepared from PC12 cells treated with 50 μM CdCl₂ for the indicated durations. The results are presented as the mean ± S.E.M. (n = 3 replicates for each group). **(C)** Immunoblots showing levels of caspase 3 and cleaved caspase 3 following treatment of PC12 cells with 50 μM CdCl₂ for up to 8 hr. **(D)** Immunoblots showing gradual changes in both SUMO-2/3 conjugation and free SUMO-2/3 levels in PC12 cells treated with 50 μM CdCl₂. **(E)** Immunoblots showing gradual changes in SENP3 levels in PC12 cells treated with 50 μM CdCl₂.

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and shRNA1 was selected for the following experiments because it resulted in the greatest decrease in endogenous SENP3 in PC12 cells (Fig. 2A). PC12 cells were transfected with either shRNA1 or a scrambled shRNA control. As a result of SENP3 knockdown, the global SUMO-2/3-ylation levels were significantly enhanced 24 hr post-transfection (Fig. 2B). After 24 hr, the transfected cells were exposed to 50 μ M CdCl₂ for another 24 hr. SENP3 knockdown significantly inhibited cadmium-induced caspase 3 cleavage (Fig. 3A, B) and reduced cell death as determined by LDH release assays (Fig. 3C). Interestingly, when we replaced the cadmium stress with another lethal factor, 0.1 μ M staurosporine (STS), neither caspase 3 cleavage nor LDH release was inhibited (Fig. 3D, E), suggesting that the SENP3 degradation-induced protective effect is specific for ROS and oxidative stress.

Next, in order to determine if increased SENP3 levels had a detrimental effect on cell survival after cadmium treatment, GFP-SENP3 was transfected in PC12 cells to achieve high SENP3 expression. However, unexpectedly there were no dramatic changes in the protein SUMO-2/3-ylation levels observed under basal conditions (Fig. 4A). In contrast to endogenous SENP3 in the PC12 cells transfected with the pEGFP vector control, high levels of both the exogenous GFP-SENP3 and endogenous SENP3 were found after a 24-hr challenge with 50 μ M CdCl₂ (Fig. 4B). In this case, higher levels of

GFP-SENP3 may overwhelm the degradation mechanism and maintain the levels of endogenous SENP3. Expression of GFP-SENP3 slightly increased caspase 3 cleavage and significantly enhanced the LDH release after 50 μ M CdCl₂ treatment for 24 hr (Fig. 4B, C). Hence, cadmium-induced cell death is elevated by increasing SENP3 levels, suggesting SENP3 loss during cadmium exposure represents a cellular protective response.

We then sought to determine whether loss of SENP3 represents a general stress response to cadmium toxicity across different cell types. To address this, we examined the response of HEK293 cells to cadmium. Similar to PC12 cells, in HEK293 cells we observed that cadmium induced concentration and time-dependent cell death as evidenced by LDH release (Fig. 5A, B), which was further confirmed by PI staining (Fig. 5C). 50 μ M CdCl₂ treatment also resulted in dynamic changes in global SUMO-2/3-ylation, which was not correlated to changes in SENP3 levels in HEK293 cells (Fig. 5D), suggesting SENP3 does not play a role in the cadmium-induced increase in protein SUMOylation observed in this model. Interestingly, 50 μ M CdCl₂ treatment for 24 hr in HEK293 cells did not result in caspase 3 cleavage, suggesting a caspase-3 independent mode of cell death in HEK293 cells (Fig. 5E, G). Furthermore, neither knocking down nor overexpressing SENP3 had an effect on either caspase 3 cleavage or LDH release in HEK293 cells challenged with 50 μ M CdCl₂ for 24 hr (Fig. 5E, F, G, H)

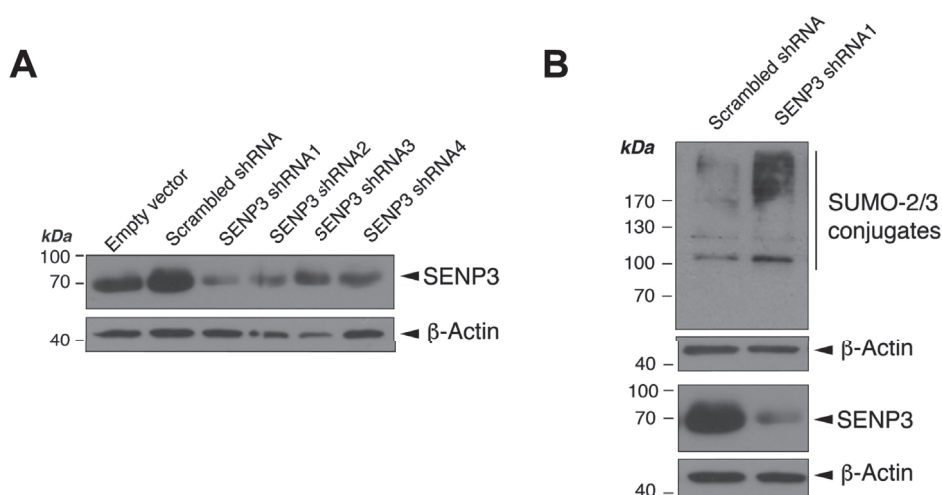


Fig. 2. SENP3 knockdown enhances global SUMO-2/3 conjugation in PC12 cells. **(A)** Immunoblots showing that transfection of four shRNAs targeting SENP3 decreased SENP3 expression levels in PC12 cells compared to those transfected with the vector control or a scrambled shRNA control. Based on efficiency in reducing SENP3 levels normalised to β -actin loading control, shRNA1 was selected for later experiments. **(B)** Immunoblots showing that SENP3 knockdown enhances global SUMO-2/3 conjugation in PC12 cells transfected with the shRNA1, compared to those transfected with the scrambled shRNA control.

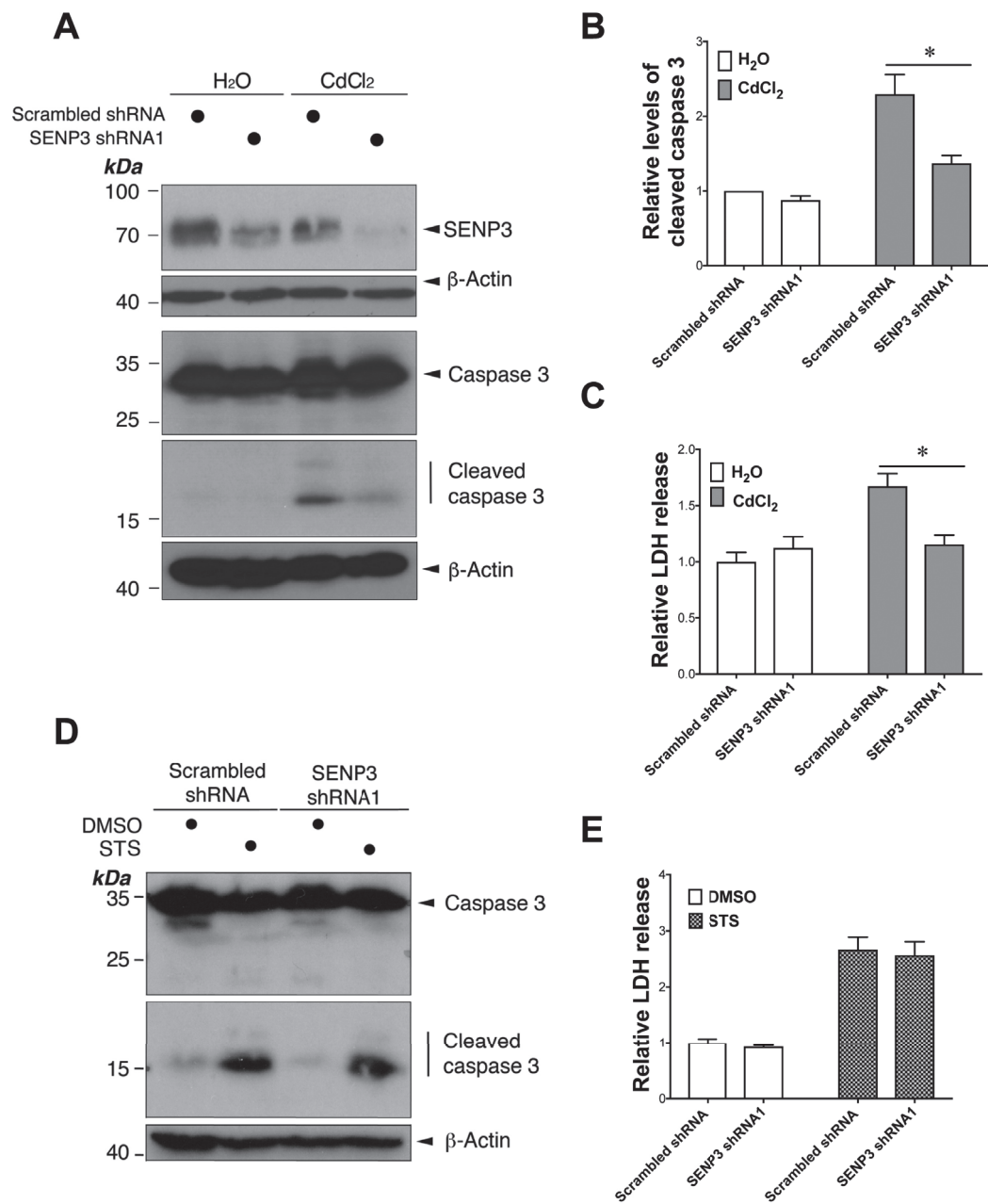


Fig. 3. SENP3 knockdown reduces caspase 3 cleavage and promotes cell survival in cadmium-treated PC12 cells. **(A)** Immunoblots showing that SENP3 knockdown markedly reduces cleaved caspase 3 levels in PC12 cells treated with 50 μ M CdCl₂ for 24 hr. **(B)** Histogram showing that SENP3 knockdown significantly reduces cleaved caspase 3 levels PC12 cells treated with 50 μ M CdCl₂ for 24 hr. The results are presented the mean \pm S.E.M. ($n = 3$ experiments using independent cell populations, $*P < 0.05$; Paired Student's t -test). **(C)** Histogram showing that SENP3 knockdown significantly reduces LDH release from PC12 cells treated with 50 μ M CdCl₂ for 24 hr. LDH levels in conditioned culture medium prepared from control and knockdown PC12 cells treated with CdCl₂ or H₂O were assessed as described in the Materials and Methods. The results are presented as mean \pm S.E.M. ($n = 3$ replicates for each group, $*P < 0.05$; Student's t -test). **(D)** Immunoblots showing that introduction of SENP3 shRNA1 into PC12 cells did not reduce cleaved caspase 3 levels induced by STS treatment (0.1 μ M; 24 hr). **(E)** Histogram showing that introduction of SENP3 shRNA1 into PC12 cells did not reduce LDH release induced by STS treatment (0.1 μ M; 24 hr). LDH levels in conditioned culture medium prepared from control and knockdown PC12 cells treated with STS or DMSO. The results are presented as mean \pm S.E.M. ($n = 3$ replicates for each group).

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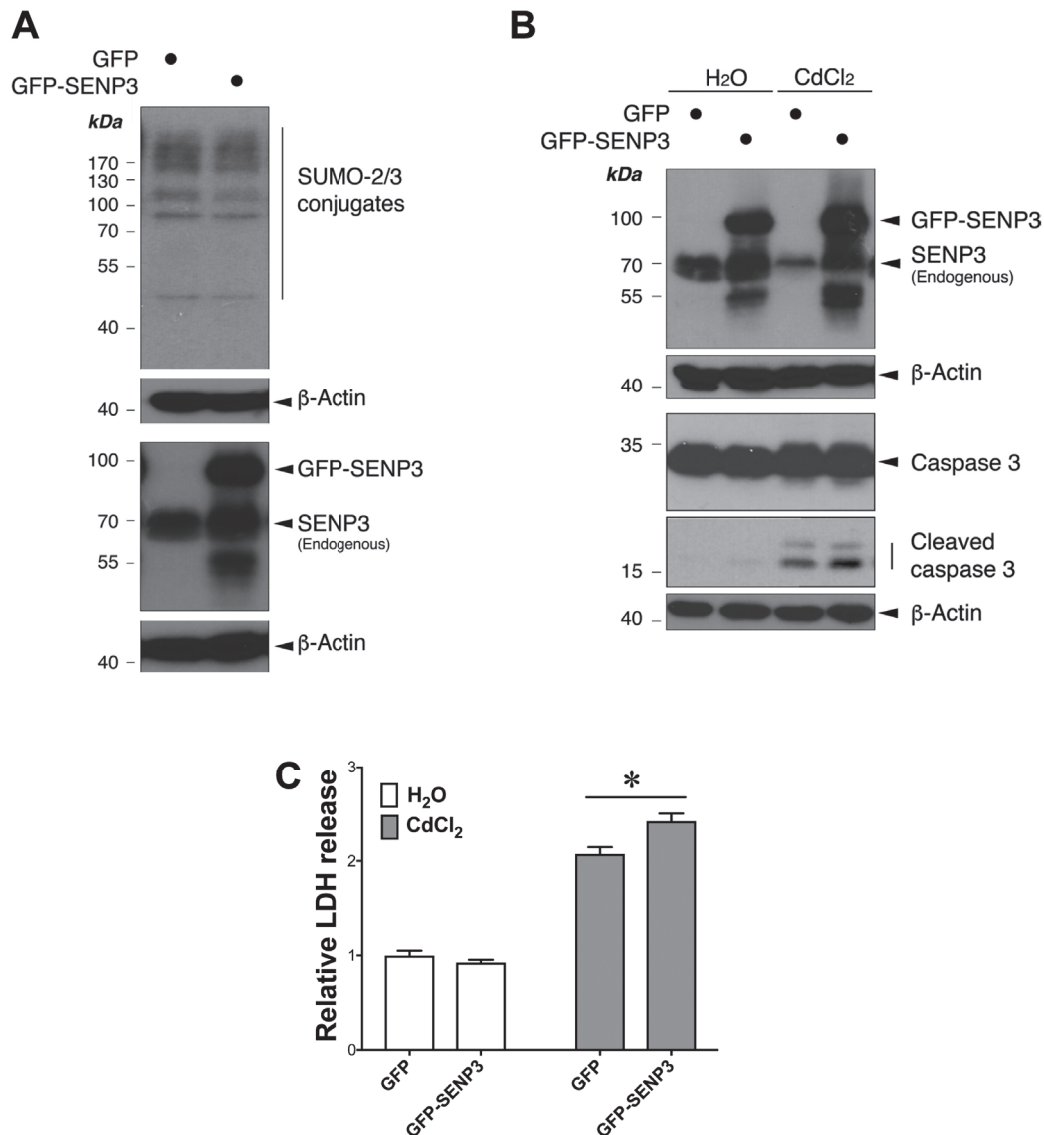


Fig. 4. SENP3 overexpression in PC12 enhances cadmium toxicity. **(A)** Immunoblots showing that expressing GFP-SEN3 in PC12 cells did not affect global SUMO-2/3 conjugation levels under basal conditions. pEGFP vector or GFP-SEN3 was transfected into PC12 cells for 24 hr followed by immunoblotting. GFP-SEN3 and endogenous SEN3 were probed with a rabbit antibody raised against SEN3. **(B)** Immunoblots showing that expressing GFP-SEN3 but not GFP increased cleaved caspase 3 levels in PC12 cells treated with 50 μ M CdCl₂ for 24 hr. **(C)** Histogram showing that expressing GFP-SEN3 but not GFP significantly increased LDH release from PC12 cells treated with 50 μ M CdCl₂ for 24 hr. LDH levels were assessed in conditioned culture medium prepared from PC12 cells expressing GFP or GFP-SEN3 and treated with cadmium or H₂O. The results are presented as mean \pm S.E.M. (n = 3 replicates for each group, *P < 0.05; Student's t-test).

suggesting that SENP3 does not play a role in the cadmium-induced caspase 3-independent cell death observed in HEK293 cells and that other components in SUMO conjugation and/or deconjugation machinery might have role(s) in this cellular process.

There is an increasing body of evidence suggesting that

SENP3 is a key regulator of stress responses (Huang *et al.*, 2009; Han *et al.*, 2010; Yan *et al.*, 2010; Wang *et al.*, 2012; Wei *et al.*, 2012; Guo *et al.*, 2013, 2017; Yang *et al.*, 2015a, 2015b; Yu *et al.*, 2015; Lee *et al.*, 2016; Guo and Henley, 2014). This study adds to previous reports by demonstrating that loss of SENP3 and the consequent

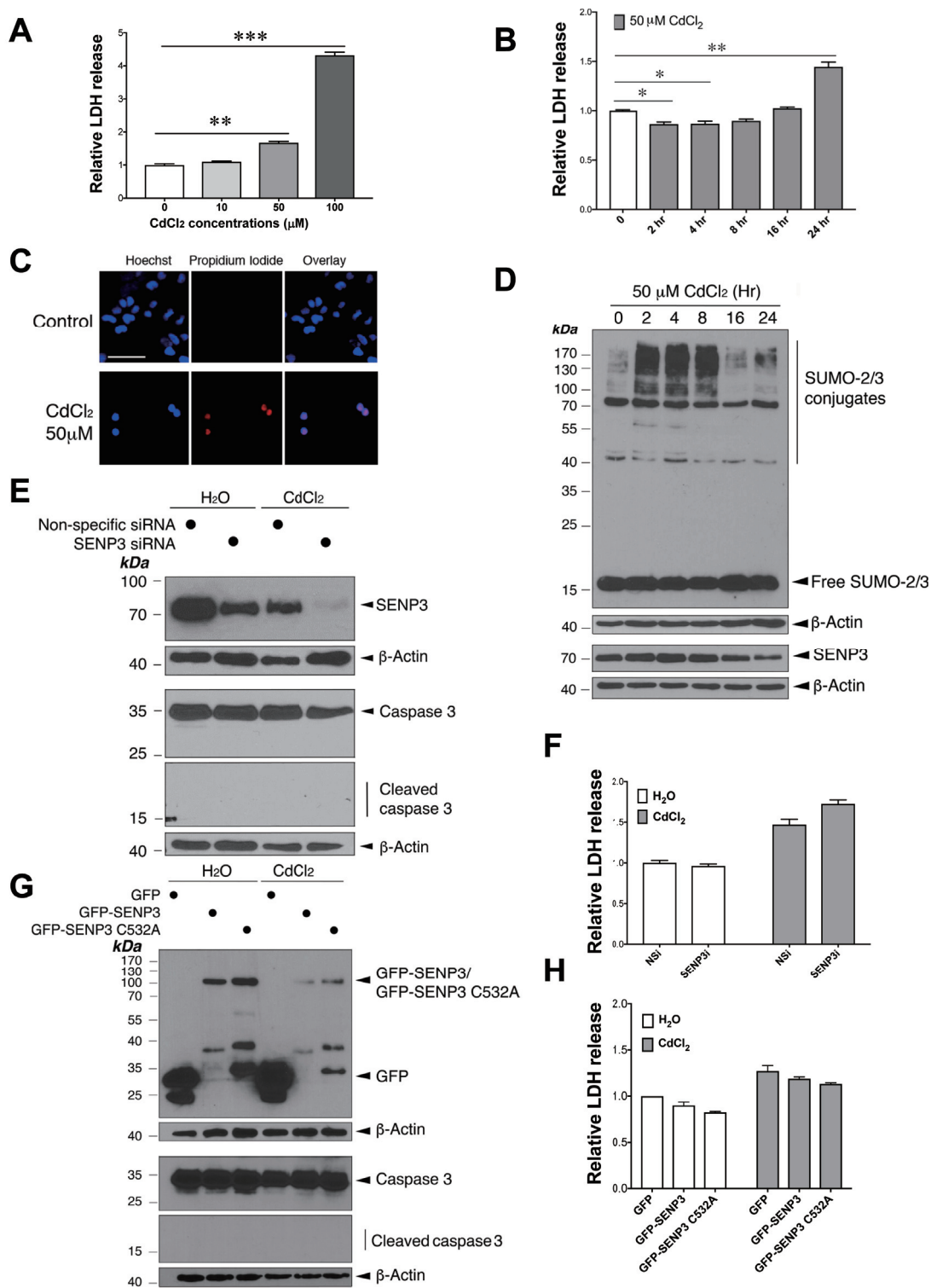


Fig. 5. SENP3 does not seem to contribute to cadmium toxicity in HEK293 cells. **(A)** Histogram showing concentration-dependent LDH release from HEK293 cells treated with cadmium for 24 hr. LDH levels were assessed in conditioned culture media prepared from HEK293 cells treated with cadmium at indicated concentrations. The results are presented as mean \pm S.E.M. ($n = 6$ replicates for each group, $**P < 0.01$ and $***P < 0.001$; one-way analysis of variance). **(B)** Histogram showing

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changes in LDH levels in conditioned culture media prepared from HEK293 cells treated with 50 μ M CdCl₂ for the indicated durations. The results are presented as mean \pm S.E.M. (n = 5 replicates for each group, *P < 0.05 and **P < 0.01; one-way analysis of variance). (C) Representative images confirming cell death in HEK293 cells treated with 50 μ M CdCl₂ by propidium iodide staining (Red: propidium iodide (PI) stain for dead cell nuclei; Blue: Hoechst for nuclear staining; scale bar: 50 μ m). (D) Immunoblots showing changes in global SUMO-2/3 conjugation, free SUMO-2/3 levels or SENP3 levels in HEK293 cells treated with 50 μ M CdCl₂ for the indicated durations. (E) Immunoblots showing absence of caspase 3 cleavage in HEK293 cells treated with 50 μ M CdCl₂ for 24 hr in the presence or absence of SENP3 knockdown using siRNA. (F) Histogram showing that SENP3 knockdown did not alter LDH release from HEK293 cells treated with 50 μ M CdCl₂ for 24 hr. LDH levels were assessed in conditioned culture medium prepared from control and SENP3 knockdown HEK293 cells treated with cadmium or H₂O. The results are presented as mean \pm S.E.M. (n = 6 replicates for each group). (G) Immunoblots showing absence of caspase 3 cleavage in HEK293 cells treated with 50 μ M CdCl₂ for 24 hr in the presence of transfected GFP, GFP-SENP3 or GFP-SENP3 C532A inactive mutant. (H) Histogram showing that expressing GFP, GFP-SENP3 or GFP-SENP3 C532A inactive mutant did not alter LDH release levels from HEK293 cells treated with 50 μ M CdCl₂ for 24 hr. LDH levels were assessed in conditioned culture medium prepared from HEK293 cells expressing GFP, GFP-SENP3 or GFP-SENP3 C532A inactive mutant and treated with cadmium or H₂O. The results are presented as mean \pm S.E.M. (n \geq 5 replicates for each group).

increase in SUMOylation plays a protective role in cadmium-induced toxicity, but that this effect is cell-type specific. Cadmium poisoning can cause widespread disruption and dysfunction of multiple cell pathways, including oxidative damage, and the mechanisms of cadmium toxicity include both caspase-dependent and independent pathways (Templeton and Liu, 2010). SENPs can exert either pro- or anti-apoptotic effects, depending on the strength, duration or type of cell stress (Pinto *et al.*, 2012; Wang *et al.*, 2012; Guo and Henley, 2014). Previously, SENP3 has been proposed to be a redox sensor because, under mild oxidative stress, its redistribution to the nucleoplasm can enhance the transcriptional activity of hypoxia-inducible factor 1 (HIF1) through deSUMOylation of its co-activator p300 (Huang *et al.*, 2009; Yan *et al.*, 2010). This redox-sensing pathway is important because SENP3 can determine cell fate following insult and injury. We have previously shown that SENP3 loss during ischaemia promotes SUMO-2/3 conjugation to Drp1, which reduces Drp1 partitioning to the mitochondrial outer membrane and inhibits its role in mitochondrial fragmentation, cytochrome *c* release, caspase activation and cell death (Guo *et al.*, 2013, 2017). During reperfusion, however, SENP3 levels recover, and the incidence of cell death was greatly increased. Interestingly, SENP3 expression has been shown to be significantly upregulated following spinal cord injury (SCI) (Wei *et al.*, 2012), subarachnoid haemorrhage (SAH) (Yang *et al.*, 2015a) and traumatic brain injury (TBI) (Yu *et al.*, 2015) in rats and mice. This is important because SCI, SAH or TBI result in both primary and secondary damage, the latter of which occurs several days after injury and involves a cascade of signalling events (Byrnes *et al.*, 2007). Correspondingly, in PC12 cells treated with hydrogen peroxide, the upregulation in SENP3 was correlated with increased cleavage of caspase

3, a marker of apoptosis, which is consistent with SENP3 upregulation playing a role in cell death following the injuries of spinal cord and brain in rats and mice (Wei *et al.*, 2012; Yang *et al.*, 2015a; Yu *et al.*, 2015). Promisingly, *in vivo* SENP3 knockdown by lentivirally expressed shRNA reduced caspase 3 cleavage and neuronal death in the cerebral cortex of a rat model for SAH (Yang *et al.*, 2015b), suggesting targeting SENP3 may constitute a novel therapeutic approach for SAH and other disorders characterised by oxidative stress and SENP3 loss.

In conclusion, we show here that regulation of SENP3 stability and consequent changes in protein SUMOylation occur in response to cadmium exposure in PC12 cells. These results elucidate a key mechanism for cell survival via enhanced protein SUMOylation mediated by the degradation of SENP3, which prevents substrate deSUMOylation in cadmium-induced caspase 3-dependent cell cytotoxicity and, very likely, other types of oxidative stress.

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Conflict of interest---- The authors declare that there is no conflict of interest.

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